Improving the potency of *N*-aryl-2,5-dimethylpyrrole antitubercular hybrid derivatives against multidrug-resistant and intracellular mycobacteria.

Meir Touitou,[a,f] Fabrizio Manetti,[b] Camila Maringolo Ribeiro,[c] Fernando Rogerio Pavan,[c] Nicolò Scalacci,[a] Katarina Zrebna,[a] Neelu Begum,[d] Dorothy Semenya,[a] Antima Gupta,[e,†] Sanjib Bhakta,[e] Timothy D. McHugh,[d] Hanoch Senderowitz,[f] Melina Kyriazi[a] and Daniele Castagnolo[a,*]

[a]School of Cancer and Pharmaceutical Sciences, King's College London, 150 Stamford Street, London SE1 9NH, UK. [b]Dipartimento di Biotecnologie, Chimica e Farmacia, via A. Moro 2, I-53100 Siena, Italy. [c]Tuberculosis Research Laboratory, School of Pharmaceutical Sciences, Sao Paulo State University, Rod. Araraquara-Jau, km1, 14800-903, Araraquara, Brazil. [d]Centre for Clinical Microbiology, University College London, London, NW3 2PF, UK. [e]Mycobacteria Research Laboratory, Department of Biological Sciences, Institute of Structural and Molecular Biology, Birkbeck, University of London , Malet Street, London WC1E 7HX, U.K. [f]Department of Chemistry, Faculty of Exact Sciences, Bar-Ilan University Ramat-Gan, 5290002 Israel.

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This manuscript is dedicated to the memory of Prof. Maurizio Botta, to whose research, teachings, vision and ability to always look beyond the obvious, we all owe much. Thank you, Prof!

ABSTRACT: A series of N-phenyl-2,5-dimethylpyrrole derivatives, designed as hybrids of the antitubercular agents BM212 and SQ109, have been synthesised and evaluated against susceptible and drug-resistant mycobacteria strains. Compound **5d**, bearing a cyclohexylmethylene side chain, showed high potency against *M. tuberculosis* including MDR-TB strains at submicromolar concentrations. The new compound shows bacteriostatic activity, low toxicity and proved to be effective against intracellular mycobacteria too, showing an activity profile similar to isoniazid.

The treatment of infectious diseases, especially those caused by bacterial pathogens such as *Mycobacterium tuberculosis* (MTB) responsible for many forms of tuberculosis (TB), is becoming a major challenge for the World Healthcare Systems.[1] During the 20th century, there was a growing confidence that infectious diseases such as TB would have been quickly annihilated thanks to the discovery of efficient antitubercular drugs[2] and the development of new therapeutic regimens which made tuberculosis a curable disease. However, the increasing ability of mycobacteria and other pathogens to adapt and develop resistance to almost all the known antibiotics undermined the initial optimism and forced us to move into the "*postantibiotic era of infectious diseases*".[3] As declared by the World Health Organisation (WHO),[4] MTB today represents a global health emergency[5] due to the emergence of multi-, extensively- and even totally-drug resistant tuberculosis (MDR-,[6] XDR-,[7] and TDR-TB,[8] respectively) strains which make the disease difficult, if not impossible, to treat with currently available drugs.[9] In addition, the ability of MTB to evade macrophage responses and to survive and replicate within those cells actually designed to kill invading microbes, makes the disease even harder to be fully eradicated.[10-12]

The standard antitubercular therapy still relies on the use of drugs introduced in the clinic in the middle of the 20th century, with the exception of the fluoroquinolones. In the last decade, only two new drugs, bedaquiline,[13] which nonetheless suffers from some major side effects,[14] and pretomanid[15] have been approved for the treatment of TB and only a limited number of drug candidates are currently undergoing clinical trials or pre-clinical development.[16]

For many years, drug discovery programmes relied heavily on target-based high-throughput screening (HTS) of large compound libraries. Nowadays, it is undeniable that such an approach suffers from limitations and that deficiencies in current compound collections have emerged, as shown by the continuing decline of success in drug discovery. With the aim to identify new anti-TB treatments, we recently adopted an alternative strategy which led to the discovery of the *N*-aryl-2,5-dimethylpyrroles **1** and **2** as hybrid derivatives of the antitubercular drugs BM212 and SQ109[17] (Figure 1).

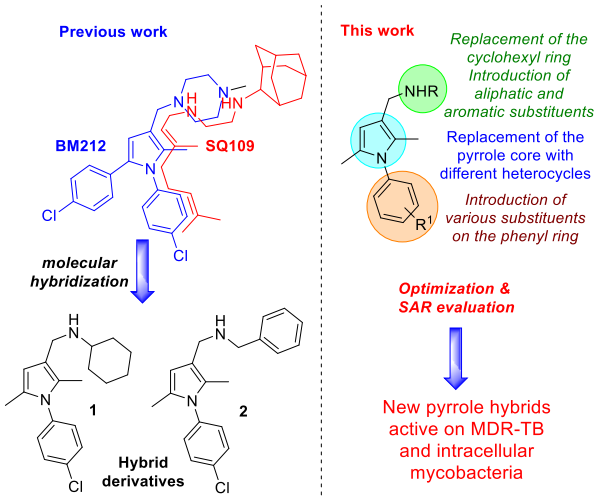
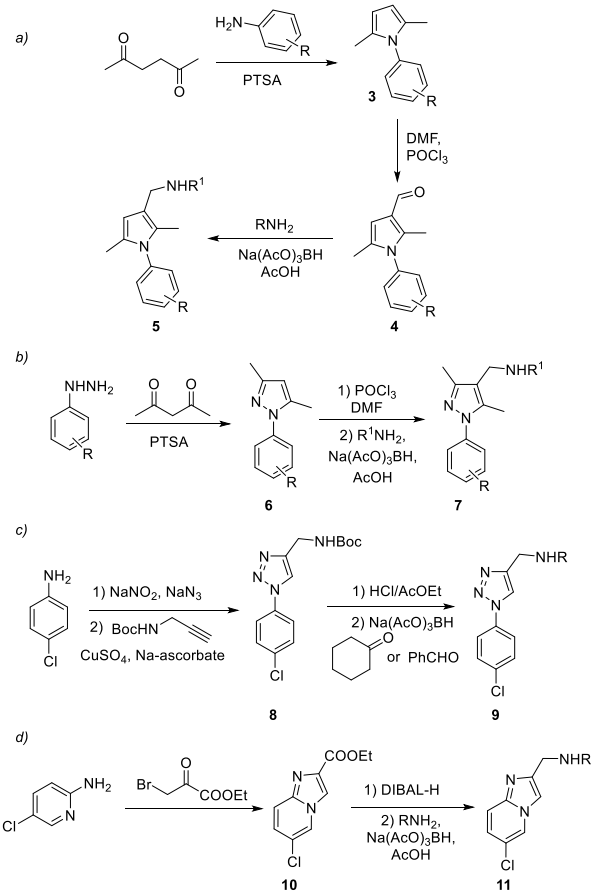


Figure 1. Pyrrole hybrid derivatives 1 and 2 and the rationale approach of the proposed work

Compounds **1-2** showed good activity against a panel of mycobacteria and an improved selectivity index (SI) compared to the parent compounds. However, in our previous work, only a limited series of pyrrole analogues were designed

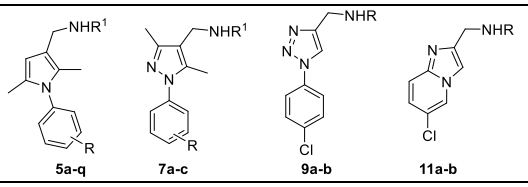
and only few structure activity relationships (SARs) were evaluated. In addition, no studies on the activity of these compounds on intra-macrophagic mycobacteria were carried out. Herein, we describe the design, synthesis and biological evaluation of a series of *N*-aryl-2,5-dimethylpyrrole derivatives with improved potency against susceptible, drug-resistant and intracellular mycobacteria. The chemical space around the 2,5-dimethylpyrrole nucleus has been explored through the replacement of the cyclohexyl and benzyl rings of **1-2** with different aromatic or aliphatic substituents. This chemical modification was carried out with the aim to confer on the new compounds a sufficient degree of lipophilicity to penetrate mycobacteria and macrophages and, in turn, inhibit intracellular mycobacteria themselves. The effect of electron-withdrawing and electron-donating substituents on the *N*-aryl ring on the antimycobacterial activity was also evaluated, as well as the role of the pyrrole nucleus. In fact, a series of analogues bearing a pyrazole, triazole and imidazopyridine rings in place of the 2,5-dimethylcore was also designed and synthesised. (Figure 1).



Scheme 1. Synthesis of antimycobacterial derivatives **5**, **7**, **9** and **11**

A series of pyrrole derivatives **5a-q** was first synthesised according to Scheme 1 (*path a*).[18] The 2,5-hexandione was reacted with different anilines to afford *N*-arylpyrroles **3**. The latter were formylated and then treated with appropriate amines in the presence of $Na(AcO)_3BH$ as reducing agent to give the desired products **5a-q** in good overall yields. Compounds **5a-d** were designed with the aim to evaluate the effect of different substituents on the C3 of the pyrrole ring other than cyclohexyl and benzyl moieties.

Table 1. Structure of antimycobacterial derivatives **5**, **7**, **9** and **11**



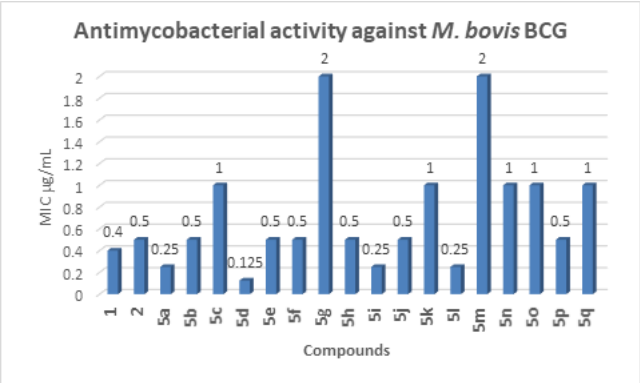
Cmpd	R	R^1
5a	Cl	2-norbornyl
5b	Cl	α-methylbenzyl
5c	Cl	phenylaminoethyl
5d	Cl	cyclohexanemethyl
5e	3,5-di-Cl	cyclohexyl
5f	2,5-di-Me	cyclohexyl
5g	2-CF_3	cyclohexyl
5h	4-F	cyclohexyl
5i	4-*i*Pr	cyclohexyl
5j	2-F	cyclohexyl
5k	4-CN	cyclohexyl
5l	4-OMe	cyclohexyl
5m	2,5-di-Me	benzyl
5n	3,5-di-Cl	benzyl
5o	4-iPr	benzyl
5p	4-F	benzyl
5q	2-CF_3	benzyl
7a	H	cyclohexyl
7b	4-Cl	cyclohexyl
7c	4-Cl	benzyl
9a	-	cyclohexyl
9b	-	benzyl
11a	-	cyclohexyl
11b	-	benzyl

For compounds **5e-q**, the side chain in C3 was left unchanged and the effects of substituents on the *N*-phenyl ring were investigated. The pyrrole ring of **1** and **2** was then replaced with different heterocyclic rings leading to derivatives **7**, **9** and **11**. Compounds **7a-c** have a pyrazole ring and were designed taking into account our previous works on antitubercular pyrazolones (Scheme 1, *path b*).[19] The reaction of phenylhydrazine and *p*-chlorophenylhydrazine with the 2,4-pentadione led to pyrazoles **6a-b** which were in turn formylated and treated with different amines under reductive amination conditions affording the desired pyrazoles **7a-c**. The pyrrole nucleus of **1-2** was then replaced by a triazole ring in derivatives **9a-b** (Scheme 1, *path c*). The *p*-chloroaniline was converted into the corresponding azide, which was then reacted with *N*-Boc-propargylamine via a Huisgen 1,3-dipolar cycloaddition reaction in the presence of $CuSO_4$ and Na-ascorbate. The resulting triazole **8** was then deprotected and reacted with benzaldehyde or cyclohexanone in the presence of $Na(AcO)_3BH$ affording desired triazole derivatives **9a-b**. Finally, the rigidified bicyclic analogues **11a-b** were designed and synthesised from 4-chloro-2-aminopyridine (Scheme 1, *path d*). This latter was reacted with ethyl bromopyruvate to afford the bicyclic ester **10** which was in turn converted into **11a-b** via DIBAL-H

reduction followed by reductive amination with the appropriate amine. The structures of the compounds **5**, **7**, **9** and **11** are reported in Table 1.

In order to quickly identify potential candidates for testing against drug resistant and intracellular mycobacteria, the compounds **5a-q** were initially screened against *M. bovis* BCG by determining the minimum inhibitory concentrations (MIC). Results are reported in Table 2. As a general trend, all the compounds **5a-q** showed good to excellent antimycobacterial activity with MIC values in the range of 0.125-2 μg/mL, thus underscoring the significance of the *N*-phenyl-2,5-dimethylpyrrole scaffold as a template for the development of antitubercular agents. SAR considerations showed that the presence of bulky, aliphatic and lipophilic substituents on the methyleneamine side chain on the C3 of the pyrrole nucleus is fundamental to improve the antimycobacterial activity. In fact, **5e-l** showed a better biological profile than the corresponding **5m-q** bearing a benzyl group in place of a cyclohexyl moiety. Also, the replacement of the cyclohexyl of **1** with the larger and more lipophilic 2-norbornyl and cyclohexanemethyl substituents led to **5a** and **5d** respectively which showed an excellent and improved activity against *M. bovis* BCG (MIC = 0.25 μg/mL for **5a** and 0.125 μg/mL for **5d**). The introduction of a more hydrophilic phenylaminoethyl chain resulted in a loss of activity in **5c** whilst **5b**, bearing an α-methylbenzyl group, showed a similar biological profile to **2**. When the chlorine atom on the *N*-aryl ring of **2** was replaced with various substituents (**5m-q**), a decrease in activity was observed. Only the derivative **5p** bearing a fluorine in place of the chlorine showed a similar biological profile (MIC = 0.5 μg/mL) to **2**. The series of compounds **5e-l**, bearing a cyclohexyl group on C3 and different substituents on the *N*-phenyl ring, showed excellent activity instead with the only exceptions of derivatives **5g** and **5k** bearing the electron-withdrawing groups -CF_3 and -CN respectively. It is evident that the electronic density on the *N*-phenyl ring has a crucial role on the antimycobacterial activity of the pyrroles **5** as discussed later.

Table 2. Preliminary screening and antimycobacterial activity of **5a-q** against *M. bovis* BCG.



These data are in perfect agreement with previous pharmacophoric speculations.[19-21] As further confirmation, the **5i** and **5l**, bearing the electron-donating substituents -*i*Pr and -OMe respectively, showed excellent MIC = 0.25 μg/mL.

Four compounds, namely **5a**, **5d**, **5i** and **5l**, were then selected from the first screening and further assayed on *M. tuberculosis* $H_{37}Rv$ as well as on clinical MDR and TB sensitive strains. The results are reported in Table 3. The clinical MDR strain CCM11.1 is identified as lineage 2 Beijing strain resistant to isoniazid, rifampicin, ethambutol, and ethionamide, while the sensitive clinical strain CCM10.1 is identified as lineage 2 Indian strain with no known resistances. Both strains were obtained from clinical isolates. Compound **5a**, **5i** and **5l** showed activity against MTB $H_{37}Rv$ similar to that observed against *M. bovis* BCG with MIC values ranging between 0.12-0.5 μg/mL. Derivative **5d**, showed an excellent profile against *M. tuberculosis* H37Rv with MIC = 0.06-0.3 μg/mL as well low cytotoxicity with an excellent Selectivity Index (SI). All the four compounds also showed excellent activity against CCM11.1 and CCM10.1 TB strains. In particular, both compound **5d** proved to be a potent inhibitor of the MDR TB strain CCM11.1 with MIC of 0.12 μg/mL and the sensitive clinical strain CCM10.1 with MIC < 0.625 ng/mL.

Table 3. Biological evaluation of compounds against bacterial and intracellular strains

Cmpd	*M. tuberculosis* MIC μg/mL			GIC_{50}	SI[b]
	H37Rv[a]	CCM 11.1[a]	CCM 10.1[a]		
5a	0.5-0.25	1	0.0025	23.4	47-94
5d	0.06[a]-0.3[c]	0.12	<0.000625	11.7	195-39
5i	0.25-0.12	0.5	0.00125	5.8	23-48
5l	0.25-0.12	0.5	<0.000625	3.9	16-32

[a]*M. tuberculosis* clinical strains from Royal Free Hospital NHS Trust, London. [b]The selectivity index is calculated as the ratio of the the GIC_{50} and the MIC observed against *M. tuberculosis* H37Rv. [c]MIC obtained from clinical strains from the Tuberculosis Research Laboratory, Sao Paulo.[22]

Finally, the non-pyrrole derivatives **7a-c**, **9a-b** and **11a-b** were tested against *M. tuberculosis* $H_{37}Rv$ strain. However, all these derivatives showed poor activity (MIC > 25 μg/mL) and were thus excluded from further studies. However, these data confirm the key role of the 2,5-dimethylpyrrole nucleus for antitubercular activity.

Encouraged by the biological profile displayed by **5d**, additional assays were conducted to assess its toxicity, and intracellular activity. The acute toxicity of **5d** *in vivo* in *Galleria mellonella* larvae was first investigated. At the administered dose of 12 mg/kg, the pyrrole **5d** elicited no apparent toxic effects on the wax moth larvae and no signs of melanisation were observed. This promising data confirmed the low toxicity of **5d** and its suitability for further development.[23] A time-kill assay was then performed in order to understand if the most active compound **5d** was bactericidal or bacteriostatic at a determined concentration (Figure 2).[24] The bactericidal activity is characterized by 3 Log_{10} CFU/mL or 99,9% reduction of the bacterial inoculum. Pyrrole **5d** presents a bacteriostatic effect at 0.32 μg/mL while the reference antibiotic moxifloxacin (MOX) did not inhibit the

MTB growth at 0.07 μg/mL but showed bactericidal effect at 0.35 μg/mL. The bactericidal and bacteriostatic effect is dependent of the concentration.

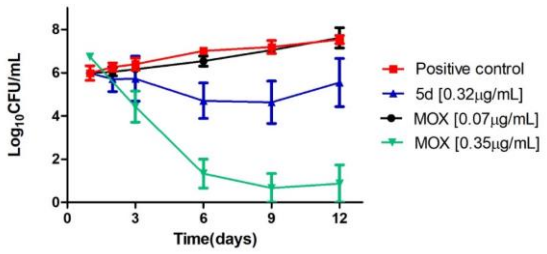


Figure 2. Time-kill of MTB $H_{37}Rv$ according time (days) in the presence of **5d** (0.32 μg/mL) or moxifloxacin (MOX – 0.07 μg/mL) or untreated (growth control).

Since the MTB is able to survive inside the macrophages, a set of experiments to evaluate the intra-macrophagic activity of **5d** was then planned. The inhibition of intracellular MTB by **5d** was initially assayed at concentrations higher or equal to MIC values and compared with those of isoniazid (INH) and moxifloxacin (MOX).[25] The concentrations tested were chosen to not be toxic to macrophages within 72 h of the assay. The data are reported in Figure 3a and clearly show that the intra-macrophage activity of **5d** is comparable to that of INH and MOX. Even if this experiment is not able to confirm if the activity of **5d** is directly based on the elimination of the bacillus once it penetrates the cellular barriers or if **5d** is able to enhance the macrophage killing action, it clearly proves the ability of **5d** in acting on intra-macrophagic mycobacteria at low concentrations.

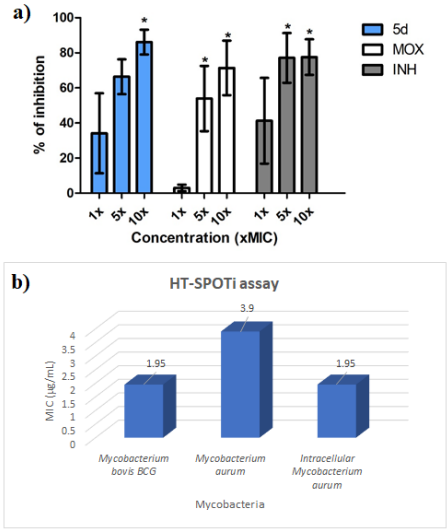


Figure 3. Figure 3a: Percent inhibition of intramacrophage MTB (Cell line J774A.1, murine origin) in the presence of **5d**, moxifloxacin (MOX) or isoniazid (INH). Concentrations: **5d** (1 x MIC = 0.32 μg/mL; 5 x MIC = 1.6 μg/mL, and 10 x MIC = 3.2 μg/mL); INH (1 x MIC = 0.06 μg/mL; 5 x MIC = 0.30 μg/mL, and 10 x MIC = 0.60 μg/mL); MOX (1 x MIC = 0.07μg/mL; 5 x MIC = 0.35μg/mL, and 10 x MIC = 0.70 μg/mL). The results are the mean and standard error of experiments performed in duplicate. * = Statistically different from the untreated control (P<0.05) by GraphPad Prism (One-way ANOVA with Dunnett's Multiple Comparison Post Test). Figure 3b: Intracellular activity of **5d** via HT-SPOTi assay

As further confirmation of the intramacrophagic activity and to estimate the intracellular MIC of compound **5d**, an intracellular HT-SPOTi assay on *Mycobacterium aurum* was then performed. The HT-SPOTi is an assay principally based on the growth of an organism on agar medium containing a range of different concentrations of drugs or inhibitors and it can be employed to screen the antimicrobial potency with high reliability and reproducibility. The *in-vitro* activity of **5d** on *M. bovis* BCG and *M. aurum* using HT-SPOTi was first investigated. The pyrrole **5d** showed good inhibition of both species at micromolar concentration (1.95 and 3.9 μg/mL respectively). The higher activity observed is related to the assay conditions which, however, is able to mimic better the conditions at which mycobacteria survive *in-vivo*. The activity of **5d** on intracellular *M. aurum* was then assayed, showing inhibition at concentration (1.95 μg/mL) lower than that of free mycobacteria (Figure 3b). This data further corroborates the potentiality of **5d** as promising antitubercular agent.

Finally, *in-silico* studies were carried out in the attempt to explain the mode of action of **5**. Both the parent compounds of hybrids **5**, namely BM212 and SQ109, have been demonstrated to inhibit MTB by binding to the mycolic acid transporter MmpL3. Due to the structural similarity between the hybrid derivatives **5** and BM212, we hypothesised a similar mode of action and, with the aim to understand their interactions with MmpL3, a molecular docking study was carried out.

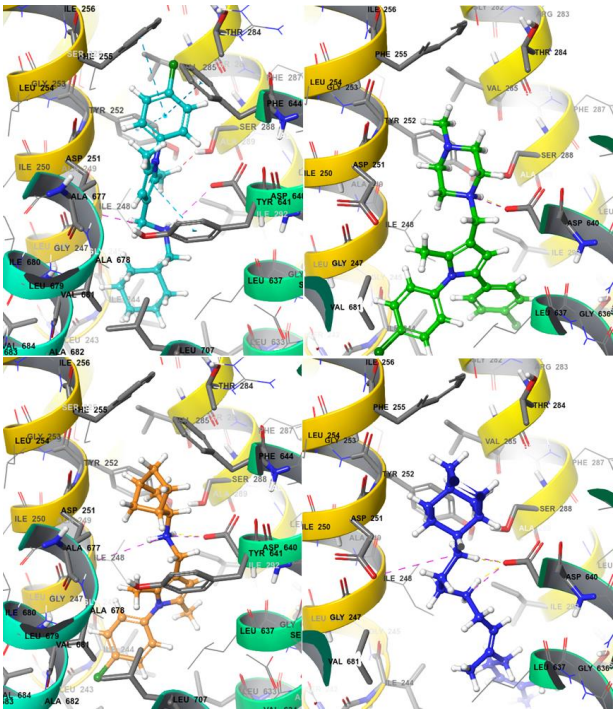


Figure 4. Binding of **5d** (up left), **5a** (down left), BM212 (up right), SQ109 (down right) with *M. tuberculosis* MmpL3 homology model

The crystal structure of MmpL3 from *M. smegmatis* is available in the RCSB Protein Data Bank (PDB)[26] and was used to generate a homology model for the MTB MmmpL3 transporter. The pyrrole derivative **5d** was thus docked into the MTB MmpL3 homology model and compared to BM212 and SQ109 binding poses. The N1 piperazine nitrogen of BM212 shows a charge-reinforced salt bridge interaction with the carboxyl terminal group of Asp640, which is known to be involved in the electrochemical proton gradient for the MmpL3 transporter.[27] On the other hand, SQ109 binds MmpL3 in a such a way that its secondary amine groups form two charge-reinforced salt bridges with the carboxyl moiety of Asp640. Similar interactions with Asp251 further stabilises the binding. Moreover, the adamantyl moiety forms hydrophobic interactions with Tyr252, Phe255 and Phe244, while the geranyl side chain interacts with Leu633, Ser295, Ala296, Ala632, and Thr314. Finally, most conformers of **5d** displayed a binding mode similar to that of BM212 with the cyclohexyl ring accommodated within the region of space where the piperazine ring of BM212 was located. In detail, the cyclohexyl ring of **5d** was embedded within the hydrophobic region constituted by Ile674, Phe644, Ile673, and Ala677. A charge-reinforced hydrogen bond between its secondary amine moiety and Asp640 is observed, as already found for BM212 and SQ109, thus suggesting this interaction of pivotal importance for binding ligands to MmpL3. In addition, the N1-chlorophenyl side chain makes hydrophobic contacts with Leu243, Ala685, Ile243, and Val684. The main structural difference between **5d** and BM212 is represented by the presence of a methyl group on C5 in place of a 4-chlorophenyl group. This simple structural modification leads **5d** to have a second binding mode in the MmpL3 binding pocket with a different orientation than BM212 as shown in Figure 4. In the inverted binding mode (Figure 4, left), the N1-chlorophenyl moiety of **5d** replaces the piperazine of BM212 in the binding pocket. This orientation of **5d** allows the pyrrole ring to have an extra π–π interaction with Tyr641, which is also reported to be involved in the electrochemical proton gradient with MmpL3 transporter. Moreover, a π–π interaction between the 4-chlorobenzene ring of **5d** and Tyr252 can be also observed in one of the MmpL3 subpocket. Tyr252 is a highly conserved residue among MmpL3 isophorms and is located with proximity to GLY253 which has been shown to mutate with exposure to SQ109 resulting in a decreased inhibition. Further reinforcement in the binding of aromatic portions of **5d** to MmpL3 is favoured by the π–π stacking interactions with Phe255, and Phe644, as well as by hydrophobic interactions with Val285 and Ala677. The secondary amine group of **5d** forms a hydrogen bond with the carbonyl group of Asp640 and Asp251. Finally, a hydrophobic subpocket of the MmpL3 binding site is able to accommodate the cyclohexyl ring of **5d** that interacts with Ile244, Leu703, Leu633, Ile248, and Gly247. The complexes formed by **5d** with the MmpL3 model provide a compelling validation of the ability of the protein to complement the binding of dimethyl pyrrole structures. Despite the lack of a second phenyl ring, the pyrrole **5d** is still capable to bind in the MmpL3 binding site. This would confirm that the presence of a second phenyl substituent plays a marginal role in the interactions of pyrrole derivatives with MmpL3. Docking simulations of **5d** have shown that it may bind to MmpL3 through two binding modes, even though with different relevance in terms of both docking scores and relative abundance. Docking calculations of **5a**, which differs from **5d** for the presence of a norbornyl side chain, were also performed. The secondary amine provides stabilisation through a hydrogen bond with Asp640 and a charge-reinforced hydrogen bond with Asp251, as found in **5d**.

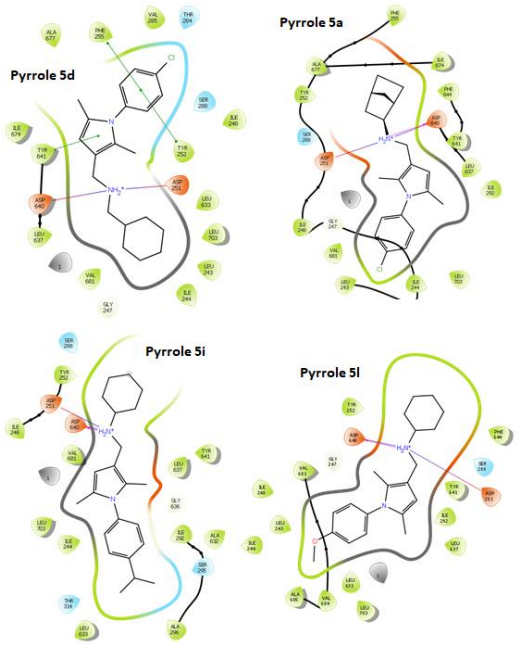


Figure 5. Binding interactions of pyrroles **5a**, **5d**, **5i** and **5l**

However, the binding mode of **5a** is inverted in respect to **5d**, with the norbornyl ring placed in the position of the 4-chlorophenyl moiety. This allows **5d** to have π–π stacking interactions with Phe255 and Tyr252 that are not observed with **5a** and can explain the higher active antitubercular activity of **5d** in comparison with **5a** and other compounds. The inverted binding mode for **5a** could be explained by the bulkier and more rigid nature of the norbornyl ring that is sterically unfavoured within the pocket defined by Leu243, Ile244, and Leu744. Docking of **5i** and **5l** showed a binding mode similar to that of **5a** with interactions between the isopropyl and methoxy groups with the hydrophobic subpocket. Figure 5. This confirms that minimal structural modifications can change the binding mode of the different pyrrole analogues, possibly contributing to modulate their antitubercular activity.

In conclusion, a new series of N-aryl-2,5-dimethylpyrrole derivatives has been synthesised and evaluated as antitubercular agents. Compound **5d**, bearing a cyclohexylmethylene side chain, showed an improved activity profile when compared to the parent drug BM212 and low toxicity, both *in vitro* as well as in preliminary *in vivo* assay on *Galleria mellonella* models. Pyrrole **5d** also showed bacteriostatic and intracellular activity against *M. tuberculosis* at concentrations similar than antibiotics moxifloxacin and isoniazid as well as inhibitory activity of the MDR strain CCM11.1. Finally, computational studies suggest that the mycolic acid transporter MmpL3 is a plausible target for **5d**,

which binds the protein similarly to BM212 and SQ109. Further studies to confirm this last hypothesis are in progress.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website. General procedures for the synthesis and biological evaluation of the compounds are reported. Full characterization of compounds **5**, **7**, **9** and **11** is reported.

AUTHOR INFORMATION

Corresponding Author

* Corresponding author. daniele.castagnolo@kcl.ac.uk

Present Addresses

† Current address: Faculty of Infectious Diseases, London School of Hygiene & Tropical Medicine, London, WC1E 7HT United Kingdom

Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

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Notes

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ABBREVIATIONS

TB, tuberculosis; MTB, *Mycobacterium tuberculosis*; MDR, multidrug resistant; XDR, extensively drug-resistant; TDR, totally drug resistant; HTS, high-throughput screening; SARs, structure activity relationships; BCG, Bacillus Calmette–Guérin; MIC, minimum inhibitory concentration; PDB, Protein Data Bank.

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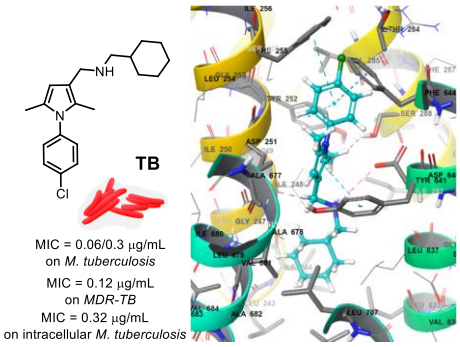
SYNOPSIS TOC (Word Style “SN_Synopsis_TOC”).



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